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Review Article

Emerging Candidate Biomarkers for Parkinson's Disease: a Review

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ABSTRACT: Parkinson's disease is a chronic neurodegenerative disorder leading to progressive motor impairment affecting more than 1% of the over-65 population. In spite of considerable progress in identifying the genetic and biochemical basis of PD, to date the diagnosis remains clinical and disease-modifying therapies continue to be elusive. A cornerstone in recent PD research is the investigation of biological markers that could help in identifying at-risk population or to track disease progression and response to therapies. Although none of these parameters has been validated for routine clinical practice yet, however some biochemical candidates hold great promise for application in PD patients, especially in the early stages of disease, and it is likely that in the future the diagnosis of PD will require a combination of genetic, imaging and laboratory data. In this review we discuss the most interesting biochemical markers for PD (including the "-omics" techniques), focusing on the methodological challenges in using *ex vivo* blood/CSF/tissue-based biomarkers and suggesting alternative strategies to overcome the difficulties that still prevent their actual use.

Key words: biomarkers; Parkinson's disease; premotor; α-synuclein; DJ-1; proteomic

Parkinson's disease (PD) is a neurodegenerative disorder estimated to affect more than 1% of the over-65 population. Despite considerable progress in identifying candidate genetic and environmental influences on the development of PD, to date the diagnosis remains mainly clinical and only confirmed at autopsy. Nevertheless, recent studies have demonstrated that a clinical misdiagnosis in the early stage of disease is not uncommon, even in the setting of a movement disorder clinic [1]. PD treatment is merely symptomatic, as clinical symptoms appear when about 70% of the dopaminergic neurons in the substantia nigra pars compacta are lost and potential disease-modifying therapies would have no effect. As a matter of fact, converging clinical, neuropathological and neuroimaging findings suggest that the neurodegenerative process in PD begins many years before the onset of motor manifestations, and the more recent or ongoing studies are largely devoted to the identification of potential markers of a preclinical or at least premotor stage of disease, in which dopaminergic neurons are relatively spared and neuroprotective treatments could have a potential effect.

The term "biomarker" was defined in 2001 by the Biomarkers Definitions Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention". This definition embraces several types of markers such as clinical quantifiable parameters, neuroimaging evaluation or biochemical assays that could help to distinguish between PD and other parkinsonisms, to reflect disease progression and to monitor the effects of therapeutic interventions.

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The identification of a premotor stage of disease through non-motor symptoms (such as olfactory deficits, constipation, rapid eye movement sleep behaviour disorder and depression) that may precede the onset of classic motor features has provided inconclusive results [2, 3]. Functional neuroimaging studies exploring the integrity of the nigrostriatal system are quite expensive and not suitable to differentiate PD from atypical parkinsonisms or to monitor the response to therapies [4]. Detection of hyperechogenicity of the substantia nigra through transcranial ultrasonography is a non-invasive technique which can reveal increased iron content in the substantia nigra of PD patients; it has been established as a valuable supplementary diagnostic marker, even in early stages of disease, but has proved to be severely limited by its operator-dependency and low specificity [5].

Given the inconclusive results obtained by clinical and neuroimaging studies, the detection of reliable biochemical markers that fulfil specific requirements of validity would be of great value. According to the purposes of the investigation, there are two main approaches to investigate biological samples for biomarkers: 1) a targeted search to detect a *a priori* defined compound in a sample or 2) an untargeted search to investigate the total load of biological components in a given body fluid or tissue. Moreover, as there are many clinical subtypes of PD, it is expected that different subgroups of PD patients could exhibit different biochemical markers; hence, a "panel" of biomarkers should be more suitable than a single marker for PD diagnosis/progression.

Although no biochemical marker can be recommended in routinary clinical practice yet, some interesting candidates exist and may prove useful in the future, alone or when analyzed in combined patterns. In this review we will analyze the most promising biochemical biomarkers for PD, focusing on the methodological challenges in using *ex vivo* biomarkers obtained from biological fluids as well as on the generation of new potential biomarkers.

α-synuclein

α-synuclein is a 140-aminoacid long protein predominantly expressed in neuronal synapses which under physiological conditions seems to play a role in the regulation of synaptic plasticity and neural differentiation. It is the major constituent of Lewy bodies, the pathological hallmark of PD and dementia with Lewy bodies (DLB). Moreover, its role in the pathogenesis of PD is strongly supported by the discovery that not only point mutations but also multiplications of the wild-type sequence of the *SNCA* gene (duplications or triplications) cause a familial form of parkinsonism. An accumulation of α-synuclein (as a consequence of failed catabolic pathways, pathogenic mutation in the *SNCA* gene or overexpression of wild-type isoform) induces a misfolding of the protein and a tendency to aggregate in oligomeric toxic compounds, hence favouring the generation of the degenerative process of PD.

α-synuclein is released from cells through a physiological secretion via the endoplasmic reticulum-Golgi complex, so extracellular α-synuclein quantification in human body fluids such as cerebrospinal fluid (CSF), plasma [6] or saliva [7] has been proposed as a potential biomarker for synucleinopathies. Nevertheless, the direct detection of extracellular α-synuclein could be hampered by the possibility of missing specific α-synuclein isoforms produced through post-translational modifications. While the measurement of monomeric α-synuclein in plasma samples provided inconclusive and contradictory results [8], recent studies showed that increased oligomeric α-synuclein levels in plasma have good specificity (85%) for detecting PD patients compared to controls [9]. Phosphorilation of α-synuclein is a key factor in the pathogenesis of PD, since 90% of α-synuclein deposited in Lewy bodies is phosphorilated at residue Ser-129 whereas only 4% of total α-synuclein in normal brain is phosphorilated. As a matter of fact, the mean level of total phosphorilated α-synuclein was found to be higher in the plasma of PD patients than controls [10]. α-synuclein levels are also detectable in peripheral blood mononuclear cells (PBMCs): our group demonstrated that, while monomeric α-synuclein raw levels are similar in PD patients and controls [11], nitrosilated α-synuclein levels are markedly increased in PBMCs of PD patients compared to healthy subjects [12].

Although PD pathology is not restricted to the central nervous system (CNS) and an interplay between central pathology and peripheral immune system is well recognised [13], it remains controversial whether alterations in peripheral samples might reflect modifications in the CNS. As CSF is close to the main site of pathology and reflects the metabolic state of the brain under varying conditions, many researchers focused their attention on CSF analysis in order to disclose reliable biomarkers of PD. The development of assays for CSF α-synuclein detection is quite recent and as many variables (such as procedure length in CSF collection, contamination with intact or lysed red blood cells, processing time, and storage conditions) could dramatically alter the outcome, a standardization of the assay is essential in order to compare results obtained by different groups. Nonetheless, most authors have shown a reduction in CSF total α-synuclein in synucleinopathies (including PD, DLB and multiple system atrophy) [14]; interestingly, the measurement of oligomeric to total CSF

α-synuclein ratio demonstrated high sensitivity and specificity for PD diagnosis [15]. The causes of decreased CSF α-synuclein are still obscure and could result from various mechanisms, such as deposition in oligomeric aggregates [14], impaired protein secretion due to progressive loss of dopaminergic cells [16] or altered translation or protein processing. Moreover, no clear correlation was observed between α-synuclein levels in the CSF and severity stages of PD [17], and possible modifications due to pharmacotherapy are still under investigation. As α-synuclein and tau are known to interact promoting mutual aggregation, it has been suggested that combined determination of these proteins and measurement of total or phosphorylated tau/α-synuclein ratios could provide a specific pattern in PD patients, contributing to diagnostic discrimination through a combination approach [18].

DJ-1

DJ-1 is a homodimeric protein involved in many cellular functions, including transcriptional regulation and response to oxidative stress, processes both critically related to neurodegeneration. Mutations in the gene encoding DJ-1 (*PARK7*) are a rare cause of autosomal recessive PD [19]. It has been reported that plasma DJ-1 levels do not significantly differ between PD patients and controls; however, direct measurement of total DJ-1 in blood is considered not suitable for PD diagnosis [20]. A very recent work explored post-translational modifications of DJ-1 in whole blood samples, disclosing higher levels of the isoforms with 4-hydroxy-2-nonenal-induced modifications in PD patients compared to AD patients and healthy controls [21].

DJ-1 is constitutively present in the CSF and several groups have examined CSF DJ-1 as a potential biomarker of PD, obtaining conflicting results: some groups demonstrated raised DJ-1 levels in the CSF of PD patients, even in early stages of disease [22], whereas in a more recent work CSF DJ-1 concentration was found instead to be decreased in PD patients compared to AD patients or healthy controls [23]. As already seen for α-synuclein, some authors tried to overcome these difficulties and better develop the diagnostic potential of DJ-1 through a combined approach: the combination of CSF α-synuclein and DJ-1 levels together with the measurements of other five molecules (namely total tau, phosphorylated tau, amyloid beta peptide 1-42, Flt3 ligand and fractalkine) lead to the detection of specific patterns in PD patients, providing high sensitivity and specificity for differentiation from other neurodegenerative diseases and tracking a correlation with disease severity and progression [17].

Of note, since CSF could not be readily obtained in most clinical settings and the precise quantification of CSF proteins can be affected by blood contamination, some authors attempted the quantification of biochemical biomarkers in other biological sources. Interestingly, α-synuclein and DJ-1 were recently identified in human saliva; indeed, submandibular gland has been recently found to be involved by synucleinopathy even at early stages of PD [24], hence the evaluation of this fluid, which is typically free of blood contamination, could provide potential and feasible diagnostic biomarkers of disease. Preliminary data showed reduced α-synuclein levels and increased DJ-1 levels in saliva obtained from PD patients compared to healthy controls [7].

Oxidative Stress Biomarkers

It is well known that oxidative stress plays a pivotal role in the pathogenesis of PD, favouring the initiation and progression of neurodegenerative processes [25]. We previously detected higher generation of reactive oxygen species (ROS) in PBMCs from PD patients treated with levodopa compared to healthy subjects. Of note, levodopa daily dosage was inversely correlated with free radical levels, suggesting that augmented oxidative stress in peripheral lymphocytes could represent a disease-related mechanism and that levodopa therapy might exert a protective effect [26]. As a confirmation, we later demonstrated the presence of increased total and mitochondrial ROS levels also in PBMCs from drug-naıve PD patients [27], consistent with previously identified mitochondrial dysfunctions including respiratory chain complex defects, induction of apoptotic signaling and oxidative damage to DNA in lymphomonocytes of PD subjects [28]. Nevertheless, ROS or nitrogen oxygen species (NOS) in peripheral samples remain unsatisfactory biomarkers, as they are not specific for PD, being found likewise altered in other neurodegenerative diseases. In addition, a variety of conditions (such as normal ageing, smoking, exercise, food, drugs…) could modulate oxidative stress levels, and control of these confounding factors could be hardly obtained.

Conversely, other indirect markers of oxidative stress such as 8-hydroxydeoxyguanosine (8-OHDG) had been demonstrated to track with good accuracy the progression of disease; moreover the increase of 8-OHDG is apparently not influenced by dopaminergic therapy [29].

The most important scavenger of free radicals in brain is the endogenous antioxidant glutathione, whose function depends on two enzymes, glutathione peroxidase (GPX) and glutathione S-transferase (GST), which are responsible for the transition from reduced to oxidized state of the molecule. As a consequence of enhanced

oxidative stress in PD, increased levels of oxidized glutathione and GST had been found not only in the substantia nigra [30] but also in peripheral blood cells of PD patients, suggesting the potential role of these antioxidant agents as reliable biomarkers for PD [31, 32].

Uric acid is another powerful antioxidant and is constitutively present in extracellular fluids, including CSF. Many evidences have demonstrated that uric acid exerts its scavenger action in many cell populations including neurons [33]. Epidemiologic studies have shown that PD risk is inversely proportional to plasma uric acid levels; moreover, among PD patients, those with higher uric acid levels in CSF were found to show a better clinical outcome and slower disease progression [34, 35]. Although uric acid is sought as a potential risk marker and a promising neuroprotective agent in PD, conflicting evidences limit its use as a diagnostic biomarker in isolation to date.

Homovanillic Acid

Since the most important hallmark of PD pathogenesis is the degeneration of dopaminergic cells in the substantia nigra, the investigation of dopaminergic metabolism and particularly of homovanillic acid (HVA) -the most important catabolite of dopamine- must be taken into account in the search for PD biomarkers. Several studies initially reported a decrease of HVA in the CSF of PD patients compared to healthy controls [36]. However, these data should be sought with caution, since they could be influenced by many biases such as the limited passage of HVA from striatum into the CSF compartment and its possible dilution by ongoing CSF production. As a matter of fact, the measurement of HVA levels in the CSF of PD patients enrolled in the DATATOP study showed great variability, limiting its use as a reliable biomarker for PD diagnosis [37].

Given the strong neurochemical interplay between dopaminergic and purinic metabolism, some authors recently combined the measurement of HVA and xanthine in the CSF, finding a strict connection between these two molecules: the [HVA]/[xanthine] ratio provided good differentiation between PD patients and controls and a correlation with disease progression, suggesting a potential role as a state and trait biomarker of PD [38].

Proteomics, metabolomics, transcriptomics

In the last decade the application of techniques capable of mass analyses (namely proteomics, metabolomics and transcriptomics, also known as the "omics" techniques) has allowed the detection of small changes in proteic or metabolic profiles in human samples such as brain tissue, plasma, urine or CSF without the use of specific antibodies, providing a metabolic "signature" of PD through an untargeted and hypothesis-free approach.

Proteomics comprises both the identification and quantification of the entire protein content (i.e. the "proteome") present in a biologic sample at a given moment. It is an unbiased approach which generates great amounts of data that can be used to compare one disease state to another. Proteomics requires the complete separation of proteins in biospecimens, subsequent analysis through mass spectrometry and quantification of proteins through advanced data processing. This approach has been applied to PD in order to obtain specific protein expression profiles that could serve as novel biomarker candidates.

Using this technique, a comprehensive characterization of the proteome in the substantia nigra was recently obtained by one group [39]. Serum does not seem a suitable biological fluid for proteomics studies, since it is characterized by extreme dynamic range which hampers an accurate and reproducible detection of candidate proteins. However, some authors applied the proteomic approach in peripheral blood cells: a panel of five proteins (cofilin 1, tropomyosin, gamma-fibrinogen, ATP synthase beta subunit and a basic actin variant) proposed to discriminate PD patients from controls was identified in PBMCs [40]. Another proteomic study demonstrated that plasma epidermal growth factor is a promising predictor of cognitive decline in PD [41].

CSF appears a more promising biofluid for proteomic studies, although blood contamination could dramatically alter CSF proteomic pattern. In a recent study, a panel of CSF proteins (including chromogranins, amyloid precursor protein-like protein 1 and prion protein) identified through a proteomic approach was suggested as a potential candidate for diagnostic purposes and disease progression tracking [42]. Likewise, in another study a more restricted set of CSF proteins (BDNF, apolipoproteins AII, apolipoprotein E, interleukin 8, A-beta 42, beta2-microglobulin, vitamin D binding protein) provided a good discriminant power for PD diagnosis [43]. Further developments will be obtained through glycoproteomics, an emerging branch of proteomics that quantifies glycoproteins in biofluids in order to identify specific profiles as potential diagnostic biomarkers.

As for proteomics, metabolomic profiling (or metabolomics) is an approach which implies that the whole metabolism, regulated by genes, exogenous substances and proteins, might be affected in neurodegenerative diseases, and that a distinct altered metabolic profile could characterize a specific disease. Metabolomics investigates end products of metabolic pathways through electrochemical coulometric array detection of low-molecular-weight molecules from biological samples followed by complex multivariative

data analysis, in order to avoid any confounding factor. This approach has been successfully applied to study potential biomarkers for various diseases, such as Huntington disease, myocardial infarction, schizophrenia, type 2 diabetes and, recently, PD: a complete separation between metabolic profiles of PD patients and controls was achieved through a metabolomic approach applied to peripheral blood samples, resulting in a significant reduction of urate levels and increase of 8-hydroxydeoxyguanosine in PD patients [44]. Other authors identified in increased pyruvate levels the main differentiator between PD and controls on metabolomic plasma samples analyses [45]. Moreover, another metabolomic study demonstrated that patients with monogenic PD due to the G2019S LRRK2 mutation show a unique metabolomic profile, distinguishable from that of sporadic PD patient and from asymptomatic carriers of the same mutation [46].

The transcriptome is the set of all RNA molecules, including mRNA, rRNA, tRNA, and other non-coding RNA produced in a population of cells; it reflects the genes that are being actively expressed at any given time. Transcriptomics investigates the levels of mRNAs in a given cell population, using high-throughput techniques based on DNA microarray technology. Unfortunately, the interpretation of relative mRNA expression levels can be complicated by the fact that small changes in mRNA expression can produce large variations in the total amount of the corresponding protein. Studies of expression profiling in cells from substantia nigra of PD patients, controls and animal models through transcriptomic approach have provided inconsistent results in regard to specific genes evaluation [47], but an overall activation of genes involved in oxidative stress, mitochondrial function or dopaminergic transmission was observed [48]. The application of transcriptomics in blood samples, where mRNA can be readily isolated, disclosed a low expression of *ST13*, a gene whose product is known to be a cofactor of heat shock protein-70, a protein critically involved in α-synuclein misfolding [49]; however, subsequent studies did not replicate these results [50].

Proteomic, metabolomic and transcriptomic profiling hold great promise for developing both diagnostic and disease progression biomarkers for PD. Nevertheless, the biomarker candidates found in these studies need further validation using more consolidated methodologies based on antibodies (such as Western blot or ELISA); moreover, "omics" methodologies require considerable technical expertise, preventing currently a large scale use.

Table 1. Candidate biochemical markers of Parkinson's disease

SOURCE	BIOMARKER	VALUE	REFERENCES
CSF	α-syn	Helpful in diagnosis of PD even at early stages; no clear correlation with severity of PD	[14], [15]
	tau/α-syn		[18]
	DJ-1		[22], [23]
	[HVA]/[xanthine]		[38]
BLOOD	α-syn	Helpful in diagnosis of PD even at early stages; poor sensitivity and/or specificity	[8], [9], [10]
	DJ-1		[20], [21]
	GST protein		[32]
	ROS		[26], [27], [28]
	Uric acid		[34], [35]
OTHERS SAMPLES	Salivary α-syn and DJ-1	More accessible biological samples; further evidences needed	[7]
	Urinary 8-OHDG		[29]
"OMICS" TECHNIQUES	Proteomics	Helpful in diagnosis of PD even at early stages; further evidences needed	[40], [42], [43]
	Metabolomics		[44], [45]
	Transcriptomics		[48], [49]

Abbreviations : α-syn, α-synuclein; GST, glutathione S-transferase ; HVA, homovanillic acid ; ROS, reactive oxygen species; 8-OHDG, 8-hydroxydeoxyguanosine

Conclusions

Although neuroprotective therapies for PD are still lacking, there is optimism that in coming years they will emerge. Hence, there is a great need of reliable markers that can be used for diagnostic purposes diagnose the disease at a preclinical stage and with a great accuracy; this could help identifying at-risk individuals who may benefit from neuroprotective or disease-modifying therapeutic strategies. Most of the current markers of

disease, such as radiolabeled tracer imaging of basal ganglia, have not been validated in preclinical stages of disease, being currently used in patients with advanced PD; moreover, they are not specific enough and are expensive. The future goal is to find reliable biomarkers of neurodegeneration in readily accessible tissues (such as peripheral blood or saliva) in order to obtain a surrogate marker of disease.

Apart from the diagnostic potential, there is also an urgent need for reliable surrogate biomarkers that could accurately track progression of disease, in order to objectively assess the magnitude and the efficacy of a symptomatic or neuroprotective treatment.

For most candidate biomarkers investigated to date, disease-associated modifications are often relatively small, with a problematic overlap between patients and controls and inconsistency between different studies. (see Table 1 above). When considering panels of various independent biomarkers, a greater diagnostic accuracy than evaluating single biomarkers was provided, suggesting that combination strategy could aid in PD diagnosis, differentiation from other conditions and correlation with disease severity and progression.

It is likely that in the future the diagnosis of PD will rest on a combination of clinical, laboratory, imaging and genetic data. The research priority will be the standardization of laboratory and imaging studies and the determination of the cheapest and most accurate combinations of tests for diagnostic purposes; the use of low-cost screening tests would help identifying candidates for more expensive and sensitive exams, such as radiolabeled tracer imaging.

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